Optimal bone mechanical and material properties require a functional glucagon-like peptide-1 receptor

Guillaume Mabileau^{1,2}, Aleksandra Mieczkowska¹, Nigel Irwin³, Peter R Flatt³ and Daniel Chappard^{1,2}

¹Groupe d'Etudes sur le Remodelage Osseux et les bioMatériaux (GEROM) – LHEA, ²Service Commun d'Imageries et d'Analyses Microscopiques (SCIAM), IRIS-IBS Institut de Biologie en Santé, LUNAM Université, CHU d'Angers, 49933 Angers Cedex, France

³School of Biomedical Sciences, Ulster University, Coleraine, UK

Correspondence should be addressed to G Mabileau

Email
guillaume.mabileau@univ-angers.fr

Abstract

Bone is permanently remodeled by a complex network of local, hormonal, and neuronal factors that affect osteoclast and osteoblast biology. Among these factors, a role for gastrointestinal hormones has been proposed based on the evidence that bone resorption dramatically falls after a meal. Glucagon-like peptide-1 (GLP1) is one of these gut hormones, and despite several reports suggesting an anabolic effect of GLP1, or its stable analogs, on bone mass, little is known about the effects of GLP1/GLP1 receptor on bone strength. In this study, we investigated by three-point bending, quantitative X-ray microradiography, microcomputed tomography, qBEI, and FTIRI bone strength and bone quality in male *Glp1r* knockout (*Glp1r* KO) mice when compared with control WT animals. Animals with a deletion of *Glp1r* presented with a significant reduction in ultimate load, yield load, stiffness, and total absorbed and post-yield energies when compared with WT animals. Furthermore, cortical thickness and bone outer diameter were significantly decreased in deficient animals. The mineral quantity and quality were not significantly different between *Glp1r* KO and WT animals. On the other hand, the maturity of the collagen matrix was significantly reduced in deficient animals and associated with lowered material properties. Taken together, these data support a positive effect of GLP1R on bone strength and quality.

Key Words

- ▶ bone quality
- ▶ glucagon-like peptide-1
- ▶ bone strength
- ▶ bone matrix
- ▶ bone mineral

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Introduction

Bone is a living mineralized material, highly complex and constantly remodeled in mass and architecture to adapt and repair the damage induced by growth, aging, and mechanical stress. In order to maintain a constant bone mass, bone remodeling necessitates a spatiotemporal coupling between osteoclasts, the bone-resorbing cells,

and osteoblasts, the bone-forming cells. Bone remodeling is a complex process regulated by various stimuli that may affect osteoclast and osteoblast physiologies. A role for the gastrointestinal tract in bone remodeling has been suggested with evidence that modulation of serum markers of bone resorption is mirrored with the profile

of gut hormone release after a meal (Henriksen *et al.* 2003, Elnenaei *et al.* 2010).

Glucagon-like peptide-1 (GLP1) is a gut hormone synthesized and secreted into the blood stream by intestinal endocrine L cells in response to a variety of stimuli (Wu *et al.* 2010). Biologically active GLP1 is secreted as a 7–37 or 7–36 amide peptide, which is rapidly inactivated to GLP1(9–36) by cleavage at the site of the second N-terminal alanine amino acid residue by the aminopeptidase, dipeptidyl peptidase IV (DPP-IV; Drucker & Nauck 2006). GLP1 action is tightly controlled by DPP-IV as more than 50% of GLP1 is inactivated before it reaches the systemic circulation and the half-life of circulating GLP1 is <2 min (Baggio & Drucker 2007). Nevertheless, GLP1 binds to its receptor, the GLP1R, widely expressed in pancreatic islets, kidney, lung, heart, stomach, intestine, and the CNS, and this interaction results in activation of intracellular signaling (Campos *et al.* 1994, Bullock *et al.* 1996).

Although uncertainty persists in clearly demonstrating the expression of GLP1R in bone cells, administration of GLP1 or its enzyme-resistant analog exendin-4 for 3 days in normal and diabetic rats results in increased trabecular bone mass and augmentation of the expression of osteoblast markers in these animals, suggesting a possible anabolic effect of GLP1 on trabecular bone (Nuche-Berenguer *et al.* 2009, 2010). Using a model of GLP1 signaling deficiency, Yamada *et al.* (2008) reported that *Glp1r* knockout mice exhibit a trend for lower trabecular bone mass in the proximal metaphysis of the tibia. These authors also reported reduction in cortical bone mineral density (BMD) as evidenced by an experimental computed tomography (CT)-based densitometry. Taken together, all these data support an anabolic role of the GLP1/GLP1R pathway in bone. However, despite these observations, little is known about the impact of the GLP1/GLP1R on bone strength. Bone strength depends on bone and mineral quantity and also on structural and material properties of the bone matrix. Microarchitecture and material properties including degree of mineralization, mineral maturity, and collagen properties are known factors that influence bone strength (Chappard *et al.* 2011).

The aims of this study were to investigate bone strength and bone quality in *Glp1r* knockout male mice. Our results suggest that *Glp1r*-deficient male mice present with significant alterations of the cortical morphology and material properties that undoubtedly resulted in reduced bone strength. These findings support a positive role for GLP1/GLP1R pathway in controlling bone strength.

Materials and methods

Animals

Sixteen-week-old male mice presenting a deletion of *Glp1r* (kindly provided by Prof D J Drucker, Toronto, Canada) were used in this study. The background and generation of *Glp1r*-deficient mice used in this study were derived from an in-house breeding colony originally described elsewhere (Hansotia *et al.* 2004). Age-matched WT mice bred in-house with the same C57BL/6 genetic background were used as controls. A total of eight deficient and 15 control mice were used in this study. Animals were maintained on a 12 h light:12 h darkness cycle in a temperature-controlled room ($21.5 \pm 1^\circ\text{C}$). Animals were individually caged and received food and water *ad libitum*. All experiments were conducted according to the UK Office regulations (UK Animals Scientific Procedures Act 1986) and European Union laws. Animals were killed by lethal inhalation of CO_2 and left and right femurs were collected, cleaned of soft tissue. The left femurs were stored in 70% ethanol at 4°C while the right femurs were frozen in a saline-soaked gauze and stored at -20°C until use. Figure 1 describes the experimental design.

Bone mechanical testing

A three-point bending experiment was performed on the right femur. Before mechanical testing, the femurs were thawed overnight and rehydrated in saline for 24 h at room temperature as described elsewhere (Ammann *et al.* 2007). Three-point bending strength was measured with a constant span length of 10 mm. The press head as well as the two support points were rounded to avoid shear load and cutting. The femurs were positioned horizontally with the anterior surface facing upward, centered on the

Figure 1

Schematic representation of the experimental design used in this study.

support, and the pressing force was applied vertically to the midshaft of the bone. Each bone was tested with a loading speed of 2 mm/min until failure with a 90 N load cell. The load–time curve obtained was converted into a load–displacement curve by the MTS TestSuite TW Software (MTS, Crétéil, France). Ultimate load and ultimate displacement were respectively defined as the maximum load and maximum displacement recorded before breakdown of the bone. Stiffness was calculated as the slope of the elastic deformation of the bone. The total absorbed energy was defined as the total area under the load–displacement curve and represents the total energy absorbed by the midshaft femur. The yield was defined as the load necessary to initiate the transformation from elastic to plastic deformation. The post-yield energy was defined as the area under the load–displacement curve from yield until failure and represents the energy absorbed by bone during plastic deformation. Material properties such as ultimate strength, ultimate strain, elastic modulus, yield strength, breaking strength, and work to fracture have been calculated from mechanical parameters previously published (Turner & Burr 1993, Ritchie *et al.* 2008).

Quantitative X-ray microradiograph imaging

Bone mineral content was determined using qXRI. Briefly, digital X-ray images of the left femur were recorded at a 12 µm pixel resolution using a Faxitron MX20 device (Edimex, Angers, France) operating at 26 kV and a 4× magnification. The region of interest was located 6 mm below the growth plate (midshaft of the femur) and represented a height of 2 mm. The relative mineral content of calcified tissues was determined as reported by Bassett *et al.* (2012) with the following modifications. Briefly, a 1.5 mm-thick steel plate, a 1.5 mm pure aluminum wire, and a 1.5 mm-thick polyester plate were used on each microradiograph and served as standards. Before converting the 16-bit DICOM images into 8-bit Tiff images, the histogram was stretched from the polyester (gray level 0) to the steel (gray level 255) standards using ImageJ 1.45s (NIH, Bethesda, MD, USA). Increasing gradations of mineralization density were represented in 16 equal intervals using the 16-color lookup table in ImageJ 1.45s. The frequency of occurrence of an *i* gray level (F_i) was calculated as follows:

$$F_i = 100 \times \frac{Ni}{Nt},$$

where Ni represents the number of pixels with the *i* gray level and Nt the total number of pixels. The frequency

distribution as a function of gray level was plotted and the mean gray level (GL_{mean}) of each bone was deduced from this distribution using the following formula:

$$GL_{\text{mean}} = \frac{\sum(F_i \times GL_i)}{100},$$

where GL_i represents the value of the *i* gray level.

X-ray microcomputed tomography

X-ray microcomputed tomography (micro-CT) analysis was performed in the proximal left femur with a Skyscan 1172 microtomograph (Bruker Micro-CT, Kontich, Belgium) equipped with an X-ray tube working at 69 kV/100 µA. The pixel size was fixed at 3.75 µm, the rotation step at 0.25°, and exposure was done with a 0.5 mm aluminum filter. The volume of interest (VOI) was located 6 mm below the growth plate. The external bone diameter (B.Dm (mm)), marrow diameter (Ma.Dm (mm)), cortical thickness (Ct.Th (µm)), and cross-sectional moment of inertia (CSMI (mm⁴)) were measured with a laboratory-based routine made using ImageJ 1.45s according to the guidelines and nomenclature proposed by the American Society for Bone and Mineral Research (Bouxsein *et al.* 2010). 3D models represent a region of interest of 2 mm centered 6 mm below the growth plate.

BMD distribution measured by backscattered electron imaging

Quantitative backscattered electron imaging was employed to determine the BMD distribution (BMDD) as previously reported (Roschger *et al.* 1998, Gaudin-Audrain *et al.* 2013). Rapidly after three-point bending, lower portions of the right femur diaphysis were embedded undecalcified in methylmethacrylate at 4 °C. Polymethylmethacrylate (PMMA) blocks were then polished to a 1 µm finish with diamond particles, carbon-coated and observed with a scanning electron microscope (EVO LS10, Carl Zeiss Ltd., Nanterre, France) equipped with a five-quadrant semiconductor backscattered electron detector. The microscope was operated at 20 keV with a probe current of 120 pA and a working distance of 8.5 mm. The backscattered signal was calibrated using pure carbon ($Z=6$, mean gray level=25), pure aluminum ($Z=13$, mean gray level=225), and pure silicon ($Z=14$, mean gray level=253) standards (Micro-analysis Consultants Ltd., St Ives, UK). For these contrast/brightness settings, the back scattered electron (BSE) gray level histogram was converted into weight percentage of calcium. Eventual changes in

brightness and contrast due to instrument instabilities were checked by monitoring the current probe and imaging the reference material (C, Al, and Si) every 15 min. The cortical bone area was imaged at a $200\times$ nominal magnification, corresponding to a pixel size of $0.5\text{ }\mu\text{m/pixel}$. The gray level distribution of each image was analyzed with a laboratory-made routine using ImageJ. Three variables were obtained from the BMDD: Ca_{peak} is the most frequently observed calcium concentration, Ca_{mean} is the average calcium concentration, and Ca_{width} is the width of the histogram at half maximum of the peak.

Nanomechanical testing

Nanoindentation tests evaluated the mechanical properties of the bone matrix. As nanoindentation assesses volume of material at a length scale less than that of individual microstructural features in bone, this technique avoids confounding factors such as bone microarchitecture and porosity that affect tissue properties at larger length scales such as three-point bending. Tests were performed on the same sample used for quantitative backscattered electron imaging (qBEI) measurements after rapid polishing. Briefly, femurs were rehydrated overnight in saline prior to nanoindentation testing. Eight indents were positioned in cortical bone 6 mm below the growth plate with a NHT-TTX System (CSM, Peseux, Switzerland) equipped with a Berkowitch diamond probe. The indents were done up to a depth of 900 nm with a loading/unloading rate of 40 mN/min. At maximum load, a holding period of 15 s was applied to avoid creeping of the bone material. Maximum load, indentation modulus, hardness, and dissipated energy were determined according to Oliver & Pharr (1992).

Fourier transformed infrared spectroscopy

After three-point bending, upper portions of the femur diaphysis were embedded in pMMA at 4°C . Sections of $4\text{ }\mu\text{m}$ thickness were cut dry on a heavy-duty microtome equipped with tungsten carbide knives (Leica Polycut S) and sandwiched between BaF_2 optical windows. Spectral analysis was obtained on a Bruker Vertex 70 spectrometer (Bruker Optics, Ettlingen, Germany) interfaced with a Bruker Hyperion 3000 infrared microscope equipped with a standard single-element Mercury Cadmium Telluride (MCT) detector ($750\text{--}4000\text{ cm}^{-1}$). Infrared spectra were recorded at a resolution of 4 cm^{-1} , with an average of 32 scans in transmission mode. Background spectral images were collected under identical conditions from the same

BaF_2 windows at the beginning and at the end of each experiment to ensure instrument stability. For Fourier transformed infrared spectroscopy (FTIR) analysis, ten spectra were acquired $\sim 6\text{ mm}$ below the growth plate on cortical bone, corresponding to the fracture zone observed by three-point bending, and analyzed with the Opus Software (release 5.5, Bruker). The contribution of the embedding pMMA and water vapor were corrected for each spectrum prior to baseline correction. Then, individual spectra have been subjected to curve fitting using a commercially available software package (Grams/AI 8.0, ThermoFisher Scientific, Villebon sur Yvette, France). Briefly, using the Levenberg–Marquardt algorithm, every absorption band is characterized by its area. The second-derivative spectrum was used to determine the number and the position of the bands constituting every spectral interval. All bands were positioned at maximal intensities with a Gaussian shape. Position, height, width at half intensity, and area were obtained. Peaks corresponding to amide I and II ($1350\text{--}1725\text{ cm}^{-1}$) and $\nu_1\nu_3$ phosphate ($900\text{--}1200\text{ cm}^{-1}$) were considered for further analysis. The evaluated IR spectral parameters were i) mineral-to-matrix ratio, which reflects the degree of mineralization of the bone matrix, calculated from the ratio of integrated areas of the phosphate ν_1, ν_3 band at $900\text{--}1200\text{ cm}^{-1}$ to the amide I band at $1585\text{--}1725\text{ cm}^{-1}$; ii) mineral maturity, which reflects the ratio of apatic vs nonapatic domains, calculated as the ratio of the relative intensity of sub-bands at 1020 and 1030 cm^{-1} of the phosphate band (Paschalis *et al.* 1996); iii) carbonate-to-phosphate ratio was expressed by the ratio of integrated areas of the $\nu_2\text{ CO}_3^{2-}$ region ($850\text{--}890\text{ cm}^{-1}$) to the ν_1, ν_3 phosphate band ($900\text{--}1200\text{ cm}^{-1}$) (Boskey *et al.* 2005); and iv) collagen maturity, determined as the relative ratio of sub-bands located at 1660 and 1690 cm^{-1} of the amide I peak. Although multiple theories about the significance of this ratio (pyridinium trivalent to dehydrodihydroxylysino-norleucine divalent collagen cross-links, modifications of secondary structure of collagen molecules) exist, this ratio indicates the maturity of the collagen of the bone matrix (Paschalis *et al.* 2001, Farlay *et al.* 2011).

Gene expression

Bone marrow cells were isolated from the long bones of 16-week-old WT animals by flushing tibias and femurs with α -MEM as previously reported (Mieczkowska *et al.* 2012). For osteoblast differentiation, bone marrow cells were plated into a 25 cm^2 culture flask until confluency (~ 3 days). For osteoclast culture, bone marrow cells were

cultured for 24 h into a 25 cm² flask in α -MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin to allow stromal cell attachment. Non-adherent cells were then collected and plated in 24-well plate in α -MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 ng/ml macrophage colony-stimulating factor (R&D Systems, Abingdon, UK), and 50 ng/ml soluble RANKL (Peprotech Ltd., London, UK). After 7 days of culture, multinucleated osteoclasts were evidenced in culture as previously described (Mabilleau *et al.* 2011). Total RNA were extracted from osteoblast or osteoclast *ex vivo* cultures, lung, pancreatic islets, and heart using TRIzol; reversed transcribed using iScript cDNA synthesis kit (Bio-Rad); and amplified by real-time PCR using SYBR GREEN PCR master mix (Bio-Rad). The mouse *Glp1r* mRNA transcript was amplified by quantitative PCR using primer pairs 5'-GGGTCTCTGGCTACATAAGGA-CAAC-3' and 5'-AAGGATGGCTGAAGCGATGAC-3'. The expression level of each sample was normalized against *Gapdh* mRNA expression using primer pairs 5'-AATG-GATTTGGACGCATTGGT-3' and 5'-TTTGCACTGGTA-CGTGTTGAT-3'.

Statistical analysis

Results were expressed as mean $\pm$ S.E.M. Non-parametric Mann-Whitney *U* test was used to compare the differences between the groups using the Systat Statistical Software release 13.0 (Systat Software, Inc., San Jose, CA, USA). Correlation between material properties and collagen cross-link ratio was assessed by a least squares linear regression analysis. Differences at $P < 0.05$ were considered significant.

Results

GLP1R is required for optimum bone strength

Bone strength of the femur was assessed by three-point bending and the results are presented Table 1. In *Glp1r* KO mice, mechanical parameters such as ultimate load, yield load, stiffness, total absorbed, and post-yield energies were reduced significantly by 17, 21, 25, 34, and 26% respectively when compared with WT animals. No differences in ultimate displacement were observed between these two groups of animals. Material properties such as work to fracture and yield strength were significantly reduced in *Glp1r*-deficient mice by 20, 20, and 7% respectively when compared with WT mice. No

Table 1 Three-point bending parameters in WT and *Glp1r* KO mice

	WT (n=12)	<i>Glp1r</i> KO (n=8)	P value
Ultimate load (n)	14.47 $\pm$ 0.35	11.95 $\pm$ 0.27*	0.034
Ultimate displacement (mm)	0.31 $\pm$ 0.006	0.30 $\pm$ 0.033	1.000
Stiffness (N/mm)	42.68 $\pm$ 0.85	31.92 $\pm$ 5.45*	0.05
Total absorbed energy (N.mm)	2.33 $\pm$ 0.09	1.54 $\pm$ 0.24*	0.021
Yield load (n)	11.83 $\pm$ 0.45	9.37 $\pm$ 0.42*	0.021
Post-yield energy (N.mm)	1.52 $\pm$ 0.05	1.12 $\pm$ 0.12*	0.048
Ultimate strength (MPa)	84.2 $\pm$ 2.0	82.9 $\pm$ 2.0	0.513
Breaking strength (MPa)	80.9 $\pm$ 3.4	79.2 $\pm$ 7.3	0.783
Ultimate strain	0.013 $\pm$ 0.001	0.011 $\pm$ 0.002	0.714
Elastic modulus (GPa)	3.0 $\pm$ 0.2	3.0 $\pm$ 0.5	0.513
Work to fracture (KJ/m ²)	0.211 $\pm$ 0.007	0.168 $\pm$ 0.017*	0.047
Yield strength (MPa)	69.3 $\pm$ 1.7	64.2 $\pm$ 0.5*	0.05

* $P < 0.05$ vs WT animals.

significant differences were observed for ultimate strength, breaking strength, ultimate strain, and elastic modulus.

Glp1r deficiency results in altered femoral mineral density and cortical geometry

As represented in Fig. 2A, the bone mineral content seemed to be lower in *Glp1r* KO animals and indeed, the frequency of occurrence of gray level, representing the bone mineral content, was switched toward the left in *Glp1r* KO mice, indicating a lower bone mineral content. Furthermore, the mean gray level was significantly reduced by 5.2% in *Glp1r* KO mice when compared with WT controls ($P = 0.021$). Histomorphometric analysis of cortical bone (Fig. 2B) revealed that B.Dm was reduced significantly by 6% in *Glp1r* KO animals when compared with WT controls ($P = 0.034$) while Ma.Dm was unchanged ($P = 0.289$). Consequently, Ct.Th and CSMI were lowered significantly by 13 and 25% respectively in deficient animals when compared with WT ($P = 0.034$ and $P = 0.031$ respectively).

Nanomechanical properties of the bone matrix are reduced in *Glp1r* KO mice

When compared with WT animals, *Glp1r*-deficient animals exhibited a significant 19% decrease in maximum load, as well as a significant reduction in bone matrix hardness (-19% ; Table 2). The indentation modulus was

Figure 2

Bone mineral content and cortical bone morphology are altered in *Glp1r* KO animals. (A) Bone mineral content as determined by quantitative X-ray microradiograph is reduced in *Glp1r* KO animals (black line and bar) as determined by a shift to the left of the frequency of occurrence of gray pixel and lower value of GL_{mean} when compared with WT (gray line and bar). (B) 3D models of WT and *Glp1r* KO femurs and (C) histomorphometric analysis of cortical bone in WT and *Glp1r* KO mice. * $P < 0.05$ vs WT animals and ** $P < 0.01$ vs WT animals.

unchanged between the two groups of animals but the energy dissipated was significantly reduced by 15% in *Glp1r*-deficient mice.

Collagen maturation but not mineral maturation is altered in *Glp1r* KO mice

The BMDD in the bone matrix was investigated by qBEI in WT and *Glp1r* KO mice (Table 3). Interestingly, no differences were observed between the two groups of animals in any of the studied parameters, indicating that the profile of mineralization of the bone matrix was similar in both cases. Similarly, the mineral-to-matrix ratio determined from FTIR measurements was not significantly different between WT and *Glp1r*^{-/-} animals (Fig. 3A). The mineral maturity and carbonate content were unchanged in *Glp1r*^{-/-} animals when compared with WT animals ($P = 0.221$ and $P = 0.536$ respectively). On the other hand, the ratio of trivalent mature over divalent immature collagen cross-links was significantly

decreased by 15% in *Glp1r*^{-/-} mice when compared with WT ($P = 0.007$, Fig. 3D). As shown in Fig. 4, a positive correlation ($R^2 = 0.62$, $P < 0.004$) was found between dissipated energy assessed by nanoindentation and the 1660:1690 cm^{-1} ratio assessed by FTIR. However, no significant correlations were found between maximum load or hardness with the 1660:1690 cm^{-1} ratio ($R^2 = 0.33$, $P = 0.067$ and $R^2 = 0.13$, $P = 0.268$ respectively).

GLP1R is not expressed in bone cells

As expected, a strong expression of GLP1R was found in lung and pancreatic islets and to a lower extent in heart (Fig. 5). On the other hand, *Glp1r* transcripts were not found in either osteoblasts or osteoclasts.

Discussion

In this study, we investigated bone strength in a mouse model of *Glp1r* deficiency to understand how the GLP1/GLP1R pathway may affect bone strength. Three-point bending experiments, performed on the midshaft femur, revealed a significant decrease in the total absorbed energy as well as reduction in ultimate load, stiffness, yield load, post-yield energy, work to fracture, and yield strength. These results suggest reduced bone strength in these animals. However, several factors might influence the outcome of three-point bending including bone microarchitecture and bone material properties (Turner & Burr 1993). Indeed, material properties calculated from stress-strain curves indicated that work to fracture and yield strength were significantly reduced in deficient animals but not ultimate strength or strain or the elastic modulus. Taken together, these data indicate that cortical bone morphology and material properties may be altered. In the C57BL/6 mouse strain, at the midshaft femur, bone tissue is composed almost exclusively of cortical bone (Judex *et al.* 2004, Buxsein *et al.* 2005). A study conducted by Yamada *et al.* (2008) reported a reduction in cortical

Table 2 Nanomechanical properties of the bone matrix

	WT (n=12)	<i>Glp1r</i> KO (n=8)	P value
Maximum load (mN)	11.9 ± 0.6	9.9 ± 0.4*	0.037
Hardness (MPa)	627.8 ± 34.4	510.7 ± 28.9*	0.028
Indentation modulus (GPa)	12.9 ± 0.6	12.1 ± 0.4	0.203
Dissipated energy (mN.mm)	3189.7 ± 176.7	2708.6 ± 130.9*	0.028

* $P < 0.05$ vs WT animals.

Table 3 Bone mineral density distribution in WT and *Glp1r*-deficient mice

	WT (n=12)	<i>Glp1r</i> KO (n=8)	P value
Ca _{peak} (%)	26.9±0.7	26.8±0.7	1.00
Ca _{mean} (%)	26.3±0.6	26.3±0.7	0.936
Ca _{width} (%)	2.7±0.1	2.9±0.2	0.337

BMD in the same *Glp1r*^{-/-} mice model in the tibia. In this study, cortical bone morphology was investigated by micro-CT and significant reductions in bone outer diameter, Ct.Th, and CSMI were observed in *Glp1r*^{-/-} mice. Interestingly, Ct.Th diminution was associated with a decrease in the outer bone diameter while the marrow diameter was unchanged. Furthermore, gene expression analysis revealed that the GLP1R is not expressed in bone cells, suggesting that the decrease in bone strength and quality observed in this animal model may be a consequence of *Glp1r* deletion in other tissues rather than a direct effect on bone cells.

Indeed, GLP1R is expressed in paraventricular, arcuate, and dorsomedial nuclei of the hypothalamus (Merchenthaler *et al.* 1999). Some of these regions have been implicated in the central control of bone remodeling by targeting osteoblasts, and as such, alterations of bone quality could also result from activation/inactivation of specific central relays. Furthermore, these mice present with a mild glucose intolerance represented by fasting hyperglycemia (Ayala *et al.* 2010). Regarding the beneficial role of insulin in bone growth and or quality, dysregulation of insulin action in these mice may also represent another mechanism leading to the observed phenotype. Furthermore, alterations of IGF1R expression have been noted in these mice (Cornu *et al.* 2009) and could also participate to the reduction in bone diameter and hence bone quality. GLP1R is also expressed in C cells of the thyroid gland and exerts, when activated by GLP1 or its stable analogs, a stimulating effect on calcitonin secretion in rodents (Bjerre Knudsen *et al.* 2010). Calcitonin is one of the most efficient inhibitors of bone resorption *in vitro* (Zaidi *et al.* 1990, Moonga *et al.* 1992). Evidence from previous studies suggests that basally, male *Glp1r* KO mice present with a 2.5-fold increase in circulating plasma calcitonin (Madsen *et al.* 2012). As regard to the inhibitory action of calcitonin on bone resorption, it would be legitimate to hypothesize that bone resorption should be the primary target of calcitonin increase. However, recent evidence made with genetic ablation of either the calcitonin or calcitonin receptor genes highlighted an

unexpected phenotype of high bone mass with no or little apparent effect on bone resorption (Hoff *et al.* 2002, Dacquin *et al.* 2004, Davey *et al.* 2008). This unexpected phenotype indicated a possible inhibitory action (direct or indirect) of calcitonin on bone formation without affecting bone resorption. Therefore, the contributing effect of increased circulating calcitonin to bone formation in our *Glp1r* KO model remains a possible scenario.

Another factor that strongly affects bone strength is the quality of bone matrix (Ammann *et al.* 2007). *Glp1r* KO mice presented with a decrease in maximum load (−17%), hardness (−19%), and dissipated energy (−15%). Owing to the nanocomposite composition of the bone matrix, any of the collagen or mineral might be involved in the reduction in material properties. Investigation of the bone mineral, by qBEI and FTIR, revealed that neither the degree of mineralization nor the maturity of the mineral was affected by the *Glp1r* deletion. However, it is important to bear in mind that due to instrument

Figure 3

FTIR assessment of bone matrix properties. (A) Mineral-to-matrix ratio, (B) mineral maturity, (C) carbonate content, and (D) collagen maturity. ***P* < 0.01 vs WT animals.

Figure 4
Regression analysis between maximum load (A), hardness (B), and dissipated energy (C) and 1660:1690 cm⁻¹ ratio. Open circles, WT and closed circles, *Glp1r* KO mice. ***P* < 0.01 vs WT.

limitations, we were not capable of investigating mineral crystallinity as proposed by Farlay *et al.* (2010). On the other hand, the maturity of the collagen matrix, determined as the 1660:1690 cm⁻¹ ratio, was significantly reduced. Some controversies exist in the interpretation of this ratio as some groups reported that this ratio represent the trivalent over divalent cross-links ratio, but recently,

an elegant study of Farlay and collaborators demonstrated that variation in this parameter could be more related to secondary structure of the collagen matrix after mineralization than a ratio of trivalent over divalent collagen cross-links (Farlay *et al.* 2011). Nevertheless, it is an indicator of collagen maturity. The reduction of collagen maturity could itself explain the reduction in material properties, as we found a significant positive association between collagen maturity and dissipated energy. Furthermore, alteration of the collagen matrix often alters the post-yield properties of the bone matrix (Bailey *et al.* 1999, Zioupos *et al.* 1999, Burr 2002). In our study, the observed reduction in mature trivalent cross-links could also account for the decreased post-yield energy determined by three-point bending.

In conclusion, mice lacking the GLP1R appear to have a decrease in bone strength observed at the anatomical level with a decrease in three-point bending resistance and decreased Ct.Th. Bone strength was also reduced at the tissue level and was associated with reductions of collagen cross-linking, but no alteration of the bone mineral. Overall, these data support a beneficial role for the GLP1/GLP1R signaling pathway in bone quality. This is important regarding the introduction of GLP1 mimetics for the treatment of patients with type 2 diabetes mellitus.

Figure 5
Expression of *Glp1r* gene in mice. RT-PCR analysis was performed using RNA prepared from lung, pancreatic islets, heart, and *ex vivo* culture of osteoblasts and osteoclasts from WT mice. Results were normalized relative to levels of *Gapdh* mRNA transcripts in the same sample. Data are mean $\pm$ S.E.M., *n* = 3.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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